RayBio® Human/Mouse/Rat Obestatin Enzyme Immunoassay Kit

Catalog #: EIA-OBS, EIAM-OBS, EIAR-OBS

User Manual
Last revised December 1, 2015

Caution:
Extraordinarily useful information enclosed

ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

Obestatin is a 24-aa peptide hormone that is produced in the stomach and small intestine of humans and several other mammals. It is encoded by the same gene that also encodes adipokine Ghrelin, which is initially expressed as a preprohormone that is subsequently cleaved into two smaller peptides, Ghrelin and Obestatin. A model has been proposed suggesting a "Yin-Yang" relationship between the two peptides, that Ghrelin and Obestatin have countering effects to each other.

There are reports using Ghrelin knockout mice studies which showed that removing the Ghrelin gene from mice did not significantly reduce their appetite. Additional reports show that Obestatin antagonizes growth hormone secretion and food intake induced by Ghrelin.

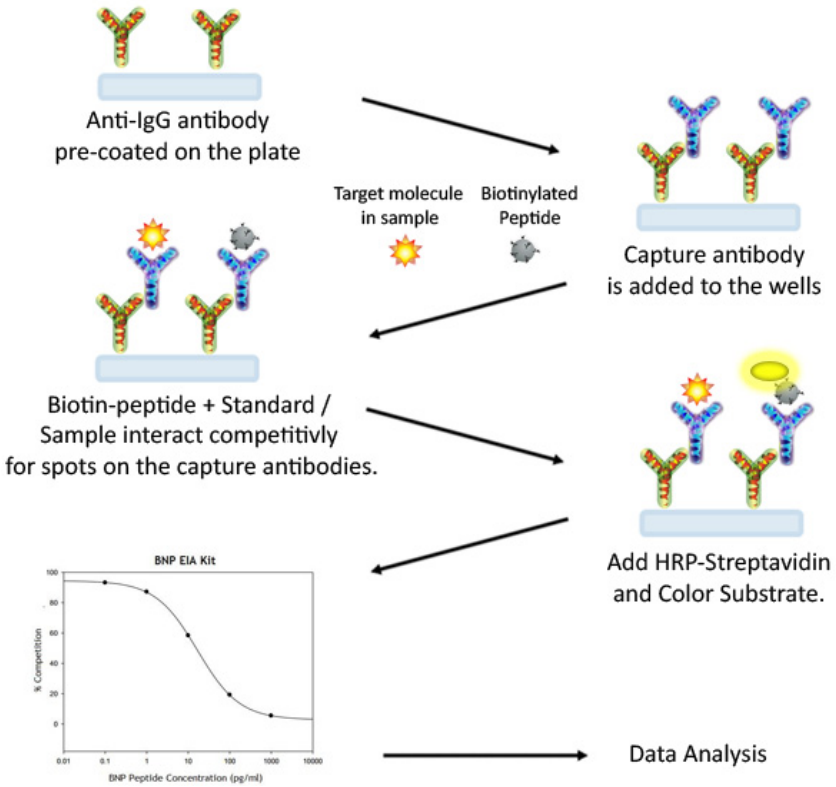
Obestatin has shown some potential clinical applications. Studies on the Obestatin/Ghrelin ratio in the gastrointestinal tract and plasma are associated with metabolic diseases such as irritable bowel syndrome, obesity and type II diabetes mellitus.

II. General Description

The RayBio® Obestatin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Obestatin peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated Obestatin peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Obestatin peptide competes with endogenous (unlabeled) Obestatin for binding to the anti-Obestatin antibody. After a wash step, any bound biotinylated Obestatin then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Obestatin peptide and inversely proportional to the amount of endogenous Obestatin in the standard or samples. A standard curve of known concentration of Obestatin peptide can be established and the concentration of Obestatin peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	**Size / Description**	**Storage / Stability After Preparation**
Obestatin Microplate (Item A)	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard Obestatin Peptide (Item C)	2 vials of Obestatin Peptide. 1 vial is enough to run each standard in duplicate.	The first standard: 2-3 days at 4°C Additional dilutions: Do not store
Anti-Obestatin Polyclonal Antibody (Item N)	2 vials of anti-Obestatin.	1 month at 4°C
Assay Diluent A (Item D)	30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma.	N/A
Assay Diluent B (Item E)	15 ml of 5X concentrated buffer. Diluent for standards, cell culture media or other sample types, and HRP-Streptavidin.	1 month at 4°C
Biotinylated Obestatin Peptide (Item F)	2 vials of Biotinylated Obestatin Peptide, 1 vial is enough to assay the whole plate.	2-3 days at 4°C
HRP-Streptavidin Concentrate (Item G)	600 µl 1000X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	1 vial of Positive Control.	2-3 days at 4°C
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Adjustable 1-25 ml pipettes for reagent preparation
4. 100 ml and 1 liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.
Note: **Assay Diluent A** should be used for dilution of samples, Item F and Item C when testing **plasma or serum samples**. **1X Assay Diluent B** should be used for dilution of samples, Item F and Item C when testing **cell culture media or other sample types**.

A. Preparation of Plate and Anti-Obestatin Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.

2. Label removable 8-well strips as appropriate for your experiment.

3. 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

4. Briefly centrifuge the anti-Obestatin antibody vial (Item N) Then add 50 µl of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently.

5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-Obestatin antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

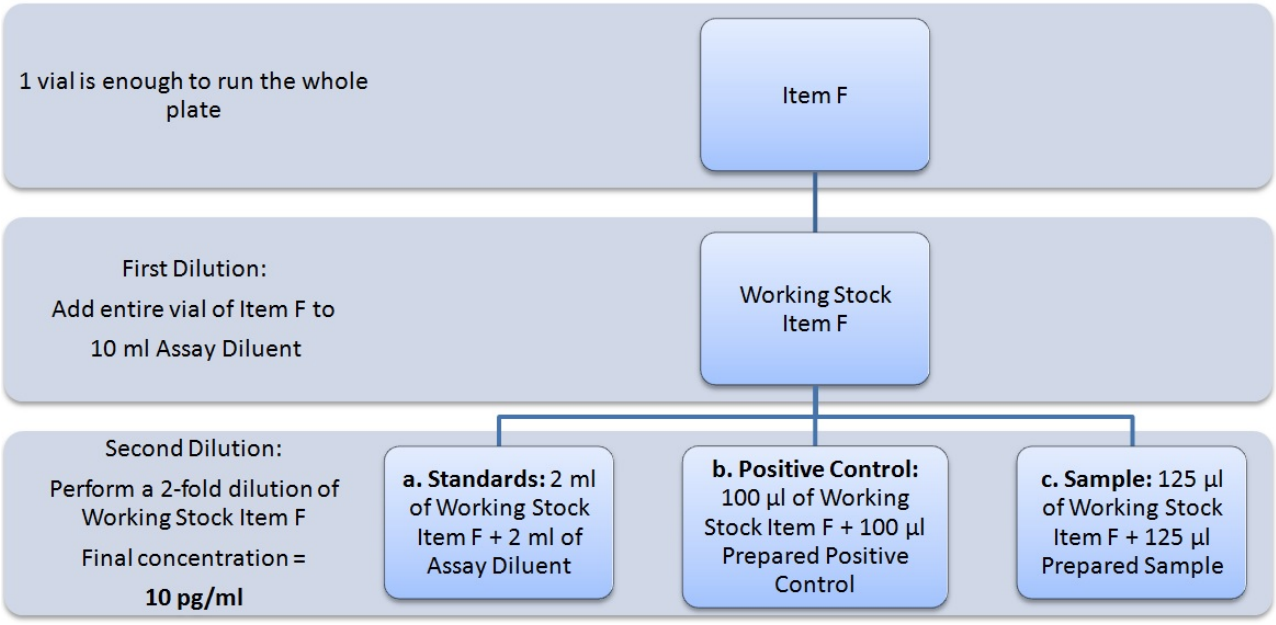
Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated Obestatin (Item F)

5. Briefly centrifuge the vial of Biotinylated Obestatin (Item F) before use.

6. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of the appropriate Assay Diluent. This is your Working Stock of Item F. Pipette up and down to mix gently. *The final concentration of biotinylated Obestatin will be **20 pg/ml***.

    a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of the appropriate Assay Diluent. The final concentration of biotinylated Obestatin will be **10 pg/ml**.
    b. Second Dilution of Item F for Positive Control: Add 100 µl of Working Stock Item F to 100 µl of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated Obestatin will be **10 pg/ml**.
    c. Second Dilution of Item F for samples: Add 125 µl of Working Stock Item F to 125 µl of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated Obestatin will be **10 pg/ml**.


1 vial is enough to run the whole plate
Item F
First Dilution:
Add entire vial of Item F to 10 ml Assay Diluent
Working Stock Item F
Second Dilution:
Perform a 2-fold dilution of Working Stock Item F
Final concentration = 10 pg/ml
a. Standards: 2 ml of Working Stock Item F + 2 ml of Assay Diluent
b. Positive Control: 100 µl of Working Stock Item F + 100 µl Prepared Positive Control
c. Sample: 125 µl of Working Stock Item F + 125 µl Prepared Sample

C. Preparation of Standards

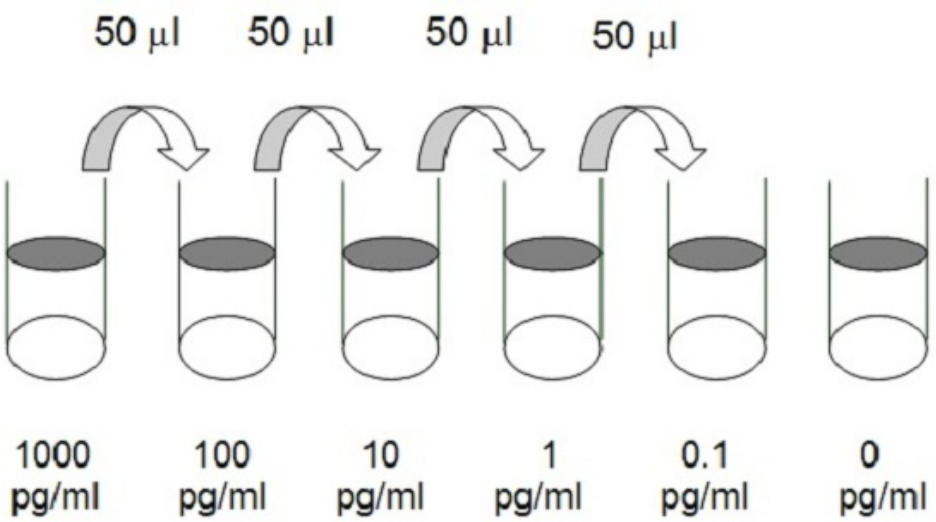
7. Label 6 microtubes with the following concentrations: 1000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated Obestatin Item F working solution (prepapred in step 6a) into each tube, except the 1,000 pg/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated Obestatin is 10 pg/ml in all standards.

8. Briefly centrifuge the vial of Obestatin Standard (Item C). Pipette 8 µl of Item C and 792 µl of 10 pg/ml biotinylated Obestatin working solution (prepared in step 6a) into the tube labeled 1000 pg/ml. Mix thoroughly. This solution serves as the first standard (1000 pg/ml Obestatin standard, 10 pg/ml biotinylated Obestatin).

9. To make the 100 pg/ml standard, pipette 50 µl of the 1000 pg/ml Obestatin standard into the tube labeled 100 pg/ml. Mix thoroughly.

10. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µl of biotinylated Obestatin and 50 µl of the prior concentration until the 0.1 pg/ml is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

11. Briefly centrifuge the Positive Control vial (Item M).

12. Refer to step 6b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Obestatin should still be 10 pg/ml.

The Positive Control is a cell culture media sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated Obestatin is 10 pg/ml.

E. Sample Preparation

13. If you wish to perform a 2-fold dilution of your sample, proceed to step 6c. If you wish to perform a higher dilution of your sample, dilute your sample with the appropriate Assay Diluent before performing step 6c.
    EXAMPLE (to make a 4-fold dilution of sample):
    a. Dilute sample 2-fold (62.5 µl of sample + 62.5 µl of the appropriate Assay Diluent.).
    b. Perform step 6c (125 µl of working solution Item F + 125 µl of sample prepared above).

    The total volume is 250 µl, enough for duplicate wells on the microplate.

It is very important to make sure the final concentration of the biotinylated Obestatin is **10 pg/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human=2X Mouse=2X Rat=2X.
If you have any questions regarding the recommendended dilutions you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

14. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.

15. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

16. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use.

17. Dilute the HRP-Streptavidin concentrate 1000-fold with 1X Assay Diluent B.

*Note: do **not** use Assay Diluent A for HRP-Streptavidin preparation in step 17*

VIII. Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µl of Anti-Obestatin Antibody (Item N) (See Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4ºC.

3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 µl each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) in appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) overnight or at 4ºC.

5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle

shaking. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3.

8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 µl anti-Obestatin to each well. Incubate 1.5 hours at room temperature or overnight at 4ºC.

3. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4ºC.

4. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

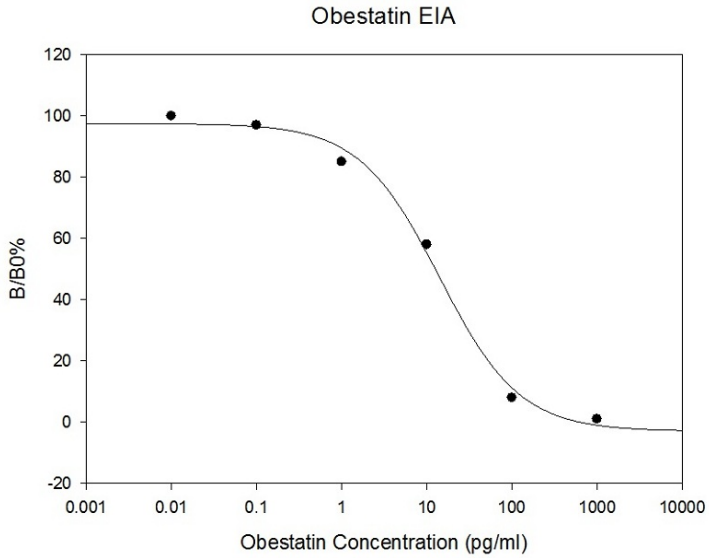
X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B-blank OD)/B_0-blank OD) where
B = OD of sample or standard and
B_0 = OD of zero standard (total binding)

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The minimum detectable concentrations of Obestatin is 0.1 pg/ml.

C. Detection Range

0.1-1,000 pg/ml

D. Reproducibility

Intra-Assay: CV<10%
Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard1	Standard1	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard2	Standard2	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard3	Standard3	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard4	Standard4	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard5	Standard5	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32	SA40	SA40

Key:
Blank = Buffer Only
Total Binding = Biotin-Obestatin only
Standard 1 = 1000 pg/ml
Standard 2 = 100 pg/ml
Standard 3 = 10 pg/ml
Standard 4 = 1 pg/ml
Standard 5 = 0.1 pg/ml
Pos Control = Biotin with Item M

XI. Specificity

Cross Reactivity: This EIA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY and APC.

XIV. Publications Citing This Product

1. Ma X., Zhao Y., Wang Q., Wu L., Wang Z., et al. Plasma Ghrelin Concentrations Are Negatively Correlated With Urine Albumin-to-Creatinine Ratio in Newly Diagnosed Type 2 Diabetes. The American Journal of Medical Sciences 2014 June. Epub Ahead of Print. doi: 10.1097/MAJ.0000000000000297
   Species: Human
   Sample Type: Plasma
2. Zhang S., et al. Ghrelin and obestatin plasma levels and ghrelin/obestatin prepropeptide gene polymorphisms in small for gestational age infants. Journal of International Medical Research published online 15 September 2014. DOI: 10.1177/0300060514533525
   Species: Human
   Sample Type: Plasma

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	• Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	• Inaccurate pipetting • Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution	• Follow storage recomendations in sections IV and V. Keep substrate solution protected from light. • Add stop solution to each well before reading plate

RayBio® ELISA Kits

Over 2,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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